Original Article
Heterogeneity of MGMT gene promoter methylation and protein expression in serial specimens in newly diagnosed glioblastoma

Qiang Pan\textsuperscript{1*}, Xue-Jun Yang\textsuperscript{3*}, Lin Zhu\textsuperscript{2*}, Chun-Yu Song\textsuperscript{1}, Xing-Tao Diao\textsuperscript{1}, Yong Gao\textsuperscript{1}, Jun Zhu\textsuperscript{1}, Xiao Yue\textsuperscript{4}, Hu-Guang Li\textsuperscript{5}, Wei-Ling Cheng\textsuperscript{6}, Yi-Ping Ning\textsuperscript{6}

Departments of \textsuperscript{1}Neurosurgery, \textsuperscript{2}ICU, Laiwu City People’s Hospital (Laiwu Hospital Affiliated to Taishan Medical College), Laiwu 271199, Shandong Province, P. R. China; \textsuperscript{3}Department of Neurosurgery, Tianjin Medical University General Hospital, Tianjin, P. R. China; \textsuperscript{4}Tianjin Huanhu Hospital, Tianjin Neurosurgery Institute, Tianjin Key Laboratory of Cerebral Vascular and Neurodegenerative Diseases, Tianjin 300052, P. R. China; Departments of \textsuperscript{5}Neurosurgery, \textsuperscript{6}ICU, Lishui People’s Hospital, Lishui 323000, Zhejiang Province, P. R. China. \textsuperscript{*}Equal contributors.

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Abstract: \textsuperscript{06}-methylguanine-DNA methyltransferase (MGMT) is known as a predictive marker, which has been associated with longer survival in glioblastoma patients who were treated with alkylating agents. However, little is known about heterogeneity of MGMT gene promoter methylation and its protein expression across different regions within the same tumor. This study was aimed to compare the status of MGMT gene promoter methylation and protein expression in different sites’ specimens of newly diagnosed glioblastoma. Two to four specimens were obtained from different regions in the same tumor during surgical procedure in 20 newly diagnosed glioblastoma patients guided by neuronavigation system (overall number of specimens: 60, all of which were prepared for the following analysis except one specimen showed necrotic obviously). MGMT promoter profile was analyzed by Methylation-specific polymerase-Chain-reaction analysis (MSP). The MGMT protein expression was assessed by immunohistochemistry (IHC) and western blotting (WB). MGMT promoter Methylation was detected in 45.76\% (27/59) specimens. IHC and WB analysis exhibited high concordant findings ($r=0.93$, $P<0.001$). There was no significant correlation between MGMT promoter methylation and protein expression (IHC and MSP, $x^2=3.18$ $P=0.074$; WB and MSP, $Z=-1.712$ $P=0.087$), and this study also demonstrated the regional heterogeneity of MGMT status of methylation in 10.00\% (2/20) patients and MGMT protein expression in 40.00\% (8/20) patients. MGMT promoter methylation is probably not the only modulating factor in MGMT protein expression. Our research questions the guiding significance of MGMT promoter methylation or its protein expression to the therapeutic scheme, which detected only in a small piece of specimen in clinical practice.

Keywords: \textsuperscript{06}-methylguanine-DNA methyltransferase, heterogeneity, gene promoter methylation, protein expression, glioblastoma multiforme

Introduction

Glioblastoma (GBM WHO IV) is the most common and malignant intracranial tumor with dismal prognosis. Its median survival time ranges from 12 to 15 months despite of aggressive surgery combined with radiation, chemotherapy [1] and biological therapy [2]. Less than 3\% of glioblastoma patients survive 5 years after initial diagnosis [3]. The highly lethal nature of this tumor partly originates from its chemoresistance of GBM tumor cells, moreover, the association between MGMT and chemoresistance is most concerned. MGMT is a ubiquitous DNA-repair enzyme in normal human tissues and many cancers. However, the expression level of MGMT varies widely [4]. In the past years, through molecular and genetic profiling efforts, the promoter hypermethylation of the MGMT gene has been shown to be a common epigenetic event in several cancers, including gliomas [5-11]. In the current study, an association of MGMT promoter methylation status, clinical outcome [12-19] and the response of tumors to alkylating agents (e.g. carmustine, lomustine, temozolomide) [16, 20] of glioblas-

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Table 1. Clinical parameters and specimen protocols of 20 patients with newly diagnosed glioblastoma

<table>
<thead>
<tr>
<th>ID</th>
<th>Age (year)</th>
<th>Sex</th>
<th>Region A</th>
<th>Region B</th>
<th>Region C</th>
<th>Region D</th>
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<tr>
<td>1</td>
<td>69</td>
<td>F</td>
<td>0.03±0.01</td>
<td>1</td>
<td>M</td>
<td>0.07±0.02</td>
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<td>N.c.</td>
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<tr>
<td>3</td>
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<td>F</td>
<td>0.50±0.08</td>
<td>2</td>
<td>U</td>
<td>N.c.</td>
<td>N.c.</td>
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<tr>
<td>4</td>
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<td>2</td>
<td>M</td>
<td>1.00±0.11</td>
<td>3</td>
</tr>
<tr>
<td>5</td>
<td>63</td>
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<td>0.16±0.01</td>
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<td>M</td>
<td>N.c.</td>
<td>N.c.</td>
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<td>F</td>
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<tr>
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<tr>
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<tr>
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<td>N.c.</td>
</tr>
<tr>
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<tr>
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<tr>
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<tr>
<td>16</td>
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<tr>
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<tr>
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<td>U</td>
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</tr>
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<td>U</td>
<td>0.36±0.05</td>
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</table>

MGMT promoter methylation (M = methylated, U = unmethylated) was assessed by Methylation-specific polymerase-Chain-reaction analysis (MSP). MGMT protein expression was evaluated by western blotting (WB) and immunohistochemistry (IHC), the level of MGMT expression assessed by WB was quantified by Densitometry and data were expressed as (mean ± SD), all tests were repeated three times (n=3); MGMT protein expression was assessed and scored by IHC in each section according to the following scale: (1 = negative or <10% positive tumor cells, 2=10%≤50%, 3≥50%). N.c.: the regional sample not collected; N.a.: sample not analyzed.

Figure 1. T1 weighted Gd-DTPA enhanced MRI image of a newly diagnosed glioblastoma from which two to four specimens were taken for analysis during operation under assistance of neuronavigation system. Letters represent the regions from which samples were taken (patient ID-10). Which were histologically confirmed to be glioblastoma.

toma patients have been revealed in some independent investigations. Based on these findings, MGMT promoter methylation is considered an ideal predictive factor for chemotherapy response and longer survival in glioblastoma. Underlying mechanisms had focused on the possible association with epigenetic silencing of the MGMT by promoter hypermethylation. However, some groups' studies failed to show any relation between MGMT promoter methylation and overall survival or response to alkylating agents [21, 22], but found prognostic significance of MGMT protein expression in glioblastoma by immunohistochemistry (IHC) [23, 24]. For example, inactivation of MGMT protein by the substrate analog O6-benzylguanine (O6-BG), sensitized tumor cells to alkylating agents [25]. The marked heterogeneity of
MGMT gene promoter methylation and its protein expression and the relationship of both remain controversial.

It is known that there is existence of histological and genetic heterogeneity in glioblastoma. Furthermore, there is no study to examine whether and to which extent MGMT protein expresses, and whether MGMT promoter methylation varies throughout the same newly diagnosed glioblastoma. If findings of heterogeneity exist, it would possibly influence diagnostic methods, clinical treatment strategies and prognostic assessments. In this study, 2-4 solid tumor samples per tumor were collected from different sites within the tumor of 20 patients with the assistance of neuronavigation system during the surgical treatment on the basis of the coregistration of magnetic resonance contrast-enhanced imaging (MRI) (3.0T EXCITE® HD, GE, American). At surgery, once the patient’s head was fixed to the Mayfield head holder, the process of registration was carried out. Multiplanar reconstructions were used in every patient for obtaining best specimens, which could highly control collecting solid tumor specimens from different sites of the tumor, and guarantee the specimens not or only minimally contaminated with tumor necrosis or non-neoplastic cells. MGMT promoter profile was analyzed by Methylation-specific polymerase-Chain-reaction analysis (MSP). The MGMT protein expression was assessed by IHC and WB. The purposes of the present prospective study are, (1) To investigate the distribution of both MGMT promoter methylation and MGMT protein expression of different sites within the tumor; (2) To explore whether MGMT protein expression and MGMT promoter methylation have intrinsic correlations; (3) To evaluate the predictive significance of MGMT promoter methylation and MGMT protein expression.

Material and methods

Patients and specimens collection

Between June 2009 and August 2010, sixty tumor specimens were obtained from twenty...
patients with newly diagnosed glioblastoma undergoing surgery at the department of Neurosurgery, Tianjin Medical University General Hospital. The clinical information relating to these patients is summarized in Table 1. Inclusion criteria were as follows, (1) Newly diagnosed glioblastoma locates in cerebral hemisphere; (2) Histopathological diagnosis of glioblastoma (WHO IV) follows the WHO criteria; (3) No chemotherapy or radiotherapy was performed on the patients before resection; (4) The study was approved by the Tianjin Medical University General Hospital Research Ethics Committee; (5) All enrolled patients have written informed the consent according to the committee’s regulations. About 2-4 tumor solid specimens were obtained from different regions within the same glioblastoma during operation under assistance of neuronavigation system (Vector Vision, BrainLAB, Germany) on the basis of the coregistration of contrast-enhanced MRI. An example is shown in Figure 1. Every specimen was divided into two pieces: one was formalin-fixed and paraffin-embedded for histological studies and IHC analysis, and the specimen was subsequently confirmed to be glioblastoma on histological evaluation. The other part immediately snap-frozen and stored in liquid nitrogen for MSP and WB analysis.

**DNA extraction and bisulfite modification of DNA**

DNA was isolated from each frozen or fresh glioblastoma tissue (Wizard® Genomic DNA Purification Kit A1120, Promega). The quantity and purity of DNA were assessed using the NanoDrop® ND-1000 Spectrophotometer (NanoDrop, Wilmington, NC). Extracted tumor DNA (1 μg) was denatured with sodium hydroxide (0.3 M), and subjected to bisulfite treatment for 18 hours at 50°C and then purified (CpGenome™ Fast DNA Modification Kit S7824, Chemicon). In this reaction, unmethylated cytosine resides, but not methylated counterpart, is modified into uracil by bisulfite treatment.

**Methylation-specific polymerase-Chain-reaction analysis, MSP assay**

DNA methylation patterns in the CpG island of MGMT (Gene Bank accession no. X-61657) was determined by MSP. Primers (5 μM) were designed for either methylated or unmethylated versions for MGMT gene according to previously described [26, 27], MGMT sense 5'-TTTGTGGTTTGTAGGTTTTTTTTTGT-3’ and MGMT antisense 5'-GCACCTCTTCCGAAAAAGAAGCG-3’ for methylated sequences; MGMT sense 5'-TTTCTGGTTTTGTAGGTTTTTTTTTGT-3’ and MGMT antisense 5'-AACCTCCACCTTCCAAA-AACAAACA-3’ for unmethylated sequences. The reaction mixture (25 μL) contained template DNA (100 ng), U or M primer (5 μM), dNTP (2.5 mM), “hot start” enzyme (1 U), with the 10× Universal PCR buffer (2.5 μL), and PCR performed under the following conditions: an initial melting step of 2 min at 95°C; followed by 36 cycles of 45 s at 95°C, 45 s at 60°C and 60 s at 72°C; and a final extension at 72°C for 8 min (CpG WIZ® MGMT Amplification Kit S7803, Chemicon). DNA from normal lymphocytes was used as negative control for methylated alleles of MGMT, and placental DNA treated in vitro with SssI methyltransferase (CpG Methyltransferase, M.SssI, M0226S, NEB) was used as positive control for methylated alleles of MGMT. The PCR products were separated on 4% agarose gels with ethidium bromide. The investigators who selected and analyzed the glioblastoma specimens were blinded all other information including clinical data and the results of IHC and WB.

**Western blotting assay**

The glioblastoma specimens were triturated in liquid nitrogen and extracted by 1% Nonidet P-40 lysis buffer. Homogenates were clarified by centrifugation at 20 000×g for 15 min at 4°C and protein concentrations were determined using a bicinchoninic acid protein assay kit (Pierce, USA). The quantity and purity of protein were also assessed using the NanoDrop® ND-1000 Spectrophotometer. Samples were adjusted to equal protein concentration and volume, and subjected to SDS-PAGE. Separate proteins were transferred to PVDF membranes (Millipore, USA) followed by blocking, The membranes were incubated with anti-MGMT antibodies (1:100 dilution, MT3.1, Chemicon) against MGMT protein, followed by incubation with HRP-conjugated secondary antibody (1:1000 dilution, Zymed). The specific protein was detected using a Super Signal protein detection kit (Pierce, USA). After being washed with stripping buffer, the membrane was reprobed with antibody against β-actin (1:100 dilution, Santa Cruz Biotechnology) using the same procedures described above. Each test was repeated in triplicates. The level of WB test of MGMT was expressed as mean integrated gray
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Figure 3. MGMT protein expression was assessed by Western blot. Top figure: representative Western blot. Bottom figure: densitometry; the data are presented as (mean ± SD). Sites A-C displayed high expression, whereas D was low or negative expression. And there are no differences among sites A-C (P>0.05), but site D is significantly lower than sites A-C (*P<0.05) (ANOVA). The test was repeated three times.

± standard deviation. The band density of specific proteins was quantified after normalization with the density of β-actin.

**MGMT immunohistochemistry assay**

Sections of formalin-fixed, paraffin-embeded tissues (6 μm thick) were dewaxed with xylenes, dehydrated by using graded ethanol and endogenous peroxidase activity was blocked by 0.3% H₂O₂ in methanol for 10 min. To unmask antigens, sections were treated with 10 mM citrate buffer (pH 6.0) at 100°C in a pressure vessel. After cooling at a room temperature, sections were washed three times with water and brought to PBS for 5 min followed by incubation with 5% BSA for 10 min. The sections incubated with appropriate primary antibody (1:200 dilution, MT3.1, Chemicon) overnight at 4°C. After being washed with PBS, biotinylated secondary antibody (1:200 dilution, Zymed) was added at a room temperature for 1 h, followed by the incubation with ABC-peroxidase for additional 1 h. After washing with Tris-buffer, the sections were incubated with DAB for 5 min, then rinsed in water and counterstained with Hematoxylin. As a control, isotyping to the primary antibody and omission of the primary antibody were done, and both gave no staining. Lymphocytes and endothelial cells served as internal positive control. MGMT was considered positive when uniform MGMT staining was detected in cell nuclei [28, 29]. Cytoplasmic only immunoreactivity and granular nuclear reactivity were considered negative [29, 30]. MGMT expression was assessed and scored in tumor cells by three pathologists who blinded to molecular and clinical data examined slides, using 3-tiered scale (1= negative or approximately limited to <10% positive tumor cells, 2=≥10%≤50% staining, 3≥50% staining) in the same tumor area from which the MSP and WB samples were tested.

**Statistical analysis**

All data are expressed as mean values ± standard deviation (x±s); the Chi-square statistics and Fisher’s exact test were used to compare MGMT expression analyzed by IHC with methylation status evaluated by MSP; the relationship between tumor MGMT protein expression analyzed by WB and MSP results was assessed by Mann-Whitney Rank Sum Test; the correlation between MGMT protein expression analyzed by IHC and by WB was assessed by Spearman Test; the MGMT protein expression of different sites within same tumor tested by WB was assessed by One-Way ANOVA. All analysis with any values blow 0.05 considered statistically significant. The statistical package SPSS 13.0 (SPSS, Inc) was used for this analysis.

**Results**

**MGMT promoter hypermethylation**

DNA was successfully extracted from tumor specimens and modified by bisulfite. All the cytosines in the unmethylated product were converted to thymines after bisulfite treatment and amplification, which suggests that the MGMT gene is unmethylated. However, the cytosines in the CpG dinucleotides of methylated product remained unchanged, as methylated cytosines cannot be modified by bisulfite, which indicated that the CpG islands of the gene are methylated. MSP products were analyzed on an agarose gel for the MGMT gene. MSP yielded interpretable results in 59 (of 60) specimens (98.33%), and one test failed due to the specimen was contaminated by necrotic tissue. MGMT promoter methylation was detected in 45.76% (27/59) specimens (Table 1).
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**MGMT protein expression**

WB and IHC assays were used to determine MGMT protein expression of all specimens (except one sample was contaminated by necrotic tissue). The level of WB test of MGMT expresses as mean integrated gray ($\bar{x} \pm s$), ranging from 0.03±0.01 to 1.66±0.12. MGMT protein was detected primarily in the nuclei by IHC test. MGMT expression was present in tumor cells with a score of 1, 2, 3, respectively in 1 (42.37%), 2 (37.29%), 3 (20.34%) specimens. MGMT protein expression level assessed by WB was well correlated with IHC ($r=0.931$, $P<0.001$). The results were shown in Table 1.

**Association between MGMT promoter methylation and its protein expression**

There was no significant correlation between MGMT promoter methylation and its protein expression: the relationship between tumor MGMT protein expression by WB and MSP results was assessed by Mann-Whitney Rank Sum Test, WB and MSP ($Z=-1.712$, $P=0.087$); The $x^2$ test and the Fisher’s exact test were used to compare MGMT expression analyzed by IHC with methylation status evaluated by MSP ($x^2=3.18$, $P=0.074$) (Table 1).

**Analysis of the series of intra-tumoral specimens**

The results of MGMT promoter methylation (evaluated by MSP) and MGMT protein expression (assessed by WB and IHC) analysis of multiple sites within the same tumor are summarized in Table 1. There are regional heterogeneity within the same tumor in 10.00% (2/20) patients’ status of methylation and 40.00% (8/20) patients’ protein expression. For example, histopathological diagnosis of four specimens from different regions (A, B, C, D) of one tumor (patient ID-10) (Figure 1) were glioblastoma (WHO IV) without obviously necrotic and normal brain tissues (Figure 2). The MGMT pro-

![Image](image_url)

**Figure 4.** MGMT protein level evaluated by IHC performed on sections from four sites within the same tumor (patient ID-10) displayed three of four sites (A-C) strong nuclear immunexpression (3), but weaker staining (1) was seen in the site (D) (DAB dyeing, original magnification: 200×). This corresponded with WB analysis.
protein level was assessed by WB showed sites A, B, C high expression, whereas D was low or no expression. There were no differences among sites A, B, C ($P > 0.05$), but site D is significantly lower than sites A, B, C ($P < 0.05$); Similarly, which was assessed by IHC displayed A, B, C strong immunoeexpression (3), but with D no immunostaining (1). MGMT promoter methylation evaluated by MSP demonstrated regions A, B, C unmethylation, whereas D was methylation, this region corresponded with MGMT protein expression assessed by WB and IHC (Table 1; Figures 3-5).

Discussion

$O^6$-methylguanine-DNA methyltransferase (MGMT) is a key enzyme in the DNA repair network, which catalyzes the transfer of the methyl group from $O^6$-methylguanine to a cysteine residue of its active site. In this single step reaction, DNA lesions caused by alkylating substances are repaired (Figure 6). MGMT subsequently is irreversibly inactivated and degraded [5-8], thus MGMT is believed to function as the utmost importance for maintaining cell integrity. MGMT gene is located on chromosome 10q26 and encodes the DNA-repair protein MGMT [31]. Nowadays, more and more studies focused on the relationship between MGMT status and chemoresistance. However, according to conflicting data having been reported [12-25], the choice of the ideal predictive marker and the appropriate detection method is still a matter of debate. In this context, it is vital to elucidate whether the MGMT promoter methylation and MGMT protein expression are homogeneously distributed or heterogeneity exists within the same tumor. Finding of heterogeneity might significantly influence the treatment strategies and prognostic judgement, and interpret the extant controversy.

In contrast to the previous studies, our study is the first investigation aimed at heterogeneity of MGMT in a series of specimens accurately collected from different regions within the tumor with the assistance of neuronavigation system in newly diagnosed glioblastomas (WHO IV), in which both tumor MGMT promoter methylation status and MGMT protein expression were simultaneously evaluated. Thus, the combination of the assistance of neuronavigation system and molecular genetic techniques might be an ideal approach for analyzing the intratumoral profile of MGMT status in glioblastoma. Our results show that the proportion of tumors exhibiting either MGMT promoter methylation...
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or MGMT protein expression did not significantly differ from previously reported studies [7, 12, 16, 27, 32-34], and MGMT protein expression level assessed by WB was well correlated with which by IHC ($r=0.931$, $P<0.001$). However, we found no significant correlation between MGMT protein expression and promoter methylation (all $P>0.05$). Moreover, this inconsistency was also observed in other neoplasms [35] and not only limited to the MGMT gene [36], so the regulation of MGMT protein expression is a more complex procedure.

Our study has also demonstrated the extent of heterogeneity of MGMT promoter methylation in 10.00% (2/20) patients and MGMT protein expression in 40.00% (8/20) patients ranging from no immunostaining to strong immunorexpression among different regions of the same tumor. Glioblastoma is known to be heterogeneous not only at the macroscopic and microscopic levels, but also at the molecular level [37, 38], which implies that this heterogeneity is a sampling error that might occur at the time of histological diagnosis, protein level evaluated by IHC and WB or promoter methylation status analyzed by MSP. In melanoma, a similar variability in MGMT promoter methylation has been reported [39].

There are a number of potential explanations for the lack of correlation between MGMT protein expression and MGMT promoter methylation, and heterogeneity of both among different regions of the same tumor, in which abnormal methylation of the promoter is not the only determining factor [40]. For example, in vitro experiments wild-type p53 seems to act as an inhibitor of MGMT expression, so that tumors with normal p53 would be more likely to have low or absent MGMT expression, independently of promoter methylation [41, 42]. On the other hand, some groups suggest that mutant p53 may be associated with decreased MGMT expression and/or methylation [37, 43], otherwise, some studies displayed loss of heterozygosity (LOH) on chromosomes 1p and 19q and MGMT inactivation were relevant [44, 45]. Lavon et al. found a significant correlation between the extent of NF-κB activation and MGMT expression in the glioma cell lines and the human glial tumors and showed that it was independent of MGMT promoter methylation. The findings of this study strongly suggest that NF-κB plays a major role in MGMT regulation [46]. In addition, all these processes of replication and cell growth, angiogenesis, apoptosis, invasion, immunomodulation change, monoallelic promoter methylation and methylation of a small proportion of malignant cells are possible influence factors.

Besides above factors, it is very practical difficulty to avoid the non-neoplastic components, such as normal glia, microglia, oligodendroglias, endothelial cells, ependymal cells, tumor infiltrating lymphocytes and CD64-positive cells et al [47] and unobvious necrosis tissue contaminated. Moreover, the limited number of patients’ specimens, variation in age and observer of test results, the differences in clinical treatment (for instance, dexamethasone treatment can upregulate both MGMT mRNA and protein levels of glioma cell lines in vitro study [48]) and so on, all above factors might also interfere with evaluation of MGMT promoter status and protein expression.

In summary, we found MGMT promoter methylation is probably not the only modulating element in MGMT protein expression. The regional heterogeneity of both MGMT promoter methylation and its protein expression in the same tumor questions the significance of the guiding role of examination as a result of one specimen collected from tumors for the choice of clinical therapy scheme. Based on our study, the results of molecular genetic and protein analysis can not yet be formally considered for making clinical decision, so it is important to keep in mind of the heterogeneity when deciding on a chemotherapeutic strategy. Whether we can analyze the MGMT status of serum [49, 50] or multi-specimens were collected from different regions with the assistance of neuronavigation system, combing with patients’ age, clinical data et al to guide alkylating agents treatment in newly diagnosed glioblastoma before we come to understand the disease better and identify biologic markers that enable us to predict prognosis and guide clinical chemotherapies, which need further evaluation in future prospective clinical trials.

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Disclosure of conflict of interest

None.

Address correspondence to: Yi-Ping Ning, Department of ICU, Lishui People’s Hospital, No. 15 Dazhong Road, Liandu Area, Lishui 323000, Zhejiang Province, P. R. China. Tel: +86-0578-2780104; E-mail: yiping_ning@yeah.net

References


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